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External and internal acetylcholinesterase in rat sympathetic neurones in vivo and in vitro

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The subcellular distribution of multiple molecular forms of acetylcholinesterase (AChE) in neurones of rat superior cervical ganglion (SCG) was determined both in vivo and in vitro by the use of selective lipid-soluble or -insoluble inhibitors. In vivo as well as in vitro, 10 S AChE is mainly outside the cell. In primary cultures of rat SCG neurones, both 4 S and 16 S AChE are mainly inside the cell. In near-term rat SCG, 4 S and 16 S are more external to the cell than in primary cultures. In adult rat SCG, 4 S AChE is equally distributed inside and outside and 16 S AChE is mainly outside the cell. Thus, specific AChE externalization probably occurs in neuronal cells as a developmentally regulated process.

Acetylcholinesterase *Molecular form* *Subcellular localization* *Adult rat superior cervical ganglion* *Near-term rat superior cervical ganglion* *Primary cell culture*

1. INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is found in many neuronal and non-neuronal tissues and can be resolved into several active molecular forms (reviews [1,2]). Although there have been many studies on the localization of AChE in muscle in vivo and in vitro [3], much less is known on the details of its biosynthesis, intracellular transport and secretion in neuronal cells. In rat superior cervical ganglia (SCG), 5 molecular forms of AChE exist in vivo as well as in vitro (4 S, 6.5 S, 10 S, 12 S and 16 S) [4,5]. The AChE sedimentation profiles differ, depending on the developmental stages reached by the neuronal cells. These forms belong to two main structural types corresponding to globular and tailed, asymmetric species first described for electric fish AChE [2,6–8]. Moreover, in addition to the collagen-like tail, other non-catalytic components may contribute to asymmetric AChE [9]. Not only globular forms [10,11] but also asymmetric forms possess hydrophilic and hydrophobic variants [12,13]. Such polymorphic features and diverse physicochemical properties suggest that AChE may interact with, and be localized in, different cell compartments or cellular structures. In this paper, we identify and describe the intracellular and extracellular pools of AChE forms in sympathetic neurons in vivo and in culture conditions.

2. MATERIALS AND METHODS

We used highly lipid-soluble and poorly lipid-soluble inhibitors of AChE: phospholine (ecothiopate iodide) (Promedica, France), a charged, poorly lipid-soluble irreversible inhibitor of external AChE, and MPT (*O*-ethyl-S_2-diisopropylaminoethylmethylphosphorothiolate) (a gift from Dr F. Leterrier, Hôpital Percy, Clamart), a highly lipid-soluble, irreversible inhibitor. BW 284 C51 (1,5 - bis(4 - allyldimethylammoniumphenyl)pentane - 3-one dibromide) (Burroughs Wellcome) is poorly lipid-soluble and reversible. It inhibits AChE outside the cell. We studied the subcellular distribution of the multiple molecular forms of rat SCG AChE in 3 different systems: in vivo, in near-term (E21; embryonic day 21) and adult SCG and in

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vitro, in 8-day-old primary cultures, obtained as previously described [5], in the presence of nerve growth factor (7 S NGF, 1 μg/ml) [14]. All incubations were performed in the culture medium [5]. In the inhibition experiments with phospholine, 10^{-5} M phospholine was applied for 3 min. After 3 washes in the basal medium, the ganglia or cells were further analyzed for AChE. In the protection experiments, 10^{-4} M BW 284 C51 was applied for 15 min, followed by BW + 1.5×10^{-7} M MPT for 15 min, and 3 washes followed, the first one in the presence of BW. Then, the ganglia were collected or cells scraped for AChE analysis. AChE activity was expressed in μmol acetylthiocholine hydrolyzed/h per ganglion or per culture dish. Sedimentation analysis was performed and quantitative data collected for the 3 main AChE peaks, i.e., 4 S + 6.5 S, 10 S + 12 S and 16 S. We have previously shown that AChE activity in such cultures is present in the sympathetic neurones themselves [5].

3. RESULTS

Phospholine or BW + MPT were applied to excised near-term (E21) rat SCG kept in basal medium as described in section 2. Table 1 gives the total AChE activities on a per ganglion basis in control, BW + MPT or phospholine-treated samples. There are roughly similar amounts of extracellular and intracellular AChE. Fig.1a shows the sedimentation patterns of AChE present in control or treated ganglia. The BW + MPT treatment results in an important decrease of 4 S AChE (4 S plus a minor 6.5 S component) and 16 S AChE (43 and 42.7% of their control activities, respectively); 10 S AChE (10 S plus a minor 12 S component) is less affected (74.3% of control activity) (table 2). In contrast, the phospholine treatment shows an important decrease of 10 S AChE (35.5% of control activity); 4 S and 16 S AChE are less affected (70.3 and 76.1% of control values, respectively). These results suggest that 10 S AChE is predominantly extracellular and 4 S and 16 S AChE are mostly internal in the neonatal SCG neurones.

The same treatments were applied to adult rat SCG. Total AChE activity was also expressed on a per ganglion basis, and the BW + MPT or phospholine treatments show that AChE is mainly extracellular (remaining activities: 60.4% after BW + MPT and 30.4% after phospholine) (table 1). Fig.1b shows the sedimentation profiles obtained in the same experiments. After BW + MPT treatment, the 4 S activity is decreased (48.6% of control), the 10 S and 16 S forms being less affected (67.0 and 70.6% of control, respectively) (table 2). The phospholine treatment differently affects the AChE forms since the activities of the 4 S, 10 S and 16 S forms represent 33.7, 24.5 and 39.7% of their control values, respectively. Thus, 4 S AChE is equally distributed in intra- and extracellular compartments and 10 S and 16 S AChE are mainly external in these adult SCG neurones.

BW + MPT or phospholine were further applied to primary cultures of neonatal SCG neurones. In 8-day-old cultures, total AChE activities expressed on a per dish basis are affected in the same manner by BW + MPT or phospholine (45.6 and 44.7% of control, respectively) indicating that the pools of extracellular and intracellular AChE are roughly the same as in the neuronal cells (table 1). Fig.1c shows the sedimentation patterns of AChE after BW + MPT, the remaining activity of 4 S, 10 S and 16 S AChE are 27.2, 56.9 and 18.7% of their control values, respectively. Correspondingly, the 4 S and 16 S forms are not affected by the phospholine treatment (116.6 and 108.3% of control values, respectively) whereas the 10 S activity is very much decreased (23.4% of remaining activity) (table 2). These results suggest that the 4 S and 16 S forms have an internal location, and that the 10 S form is more external in in vitro conditions.

Table 1

Effect of inhibitors on total AChE activity in near-term (E21) and adult rat SCG and in SCG primary cultures

	Control AChE activity (μmol/h per ganglion or dish)	BW + MPT AChE activity (% of control)	Phospholine AChE activity (% of control)
E21	353 ± 37 $n = 4$	53.7 ± 4.1 $n = 4$	61.3 ± 6.4 $n = 4$
Adult	4660 ± 243 $n = 4$	60.4 ± 5.0 $n = 5$	30.4 ± 6.4 $n = 5$
Culture	1132 ± 81 $n = 9$	45.6 ± 5.6 $n = 10$	44.7 ± 3.5 $n = 7$

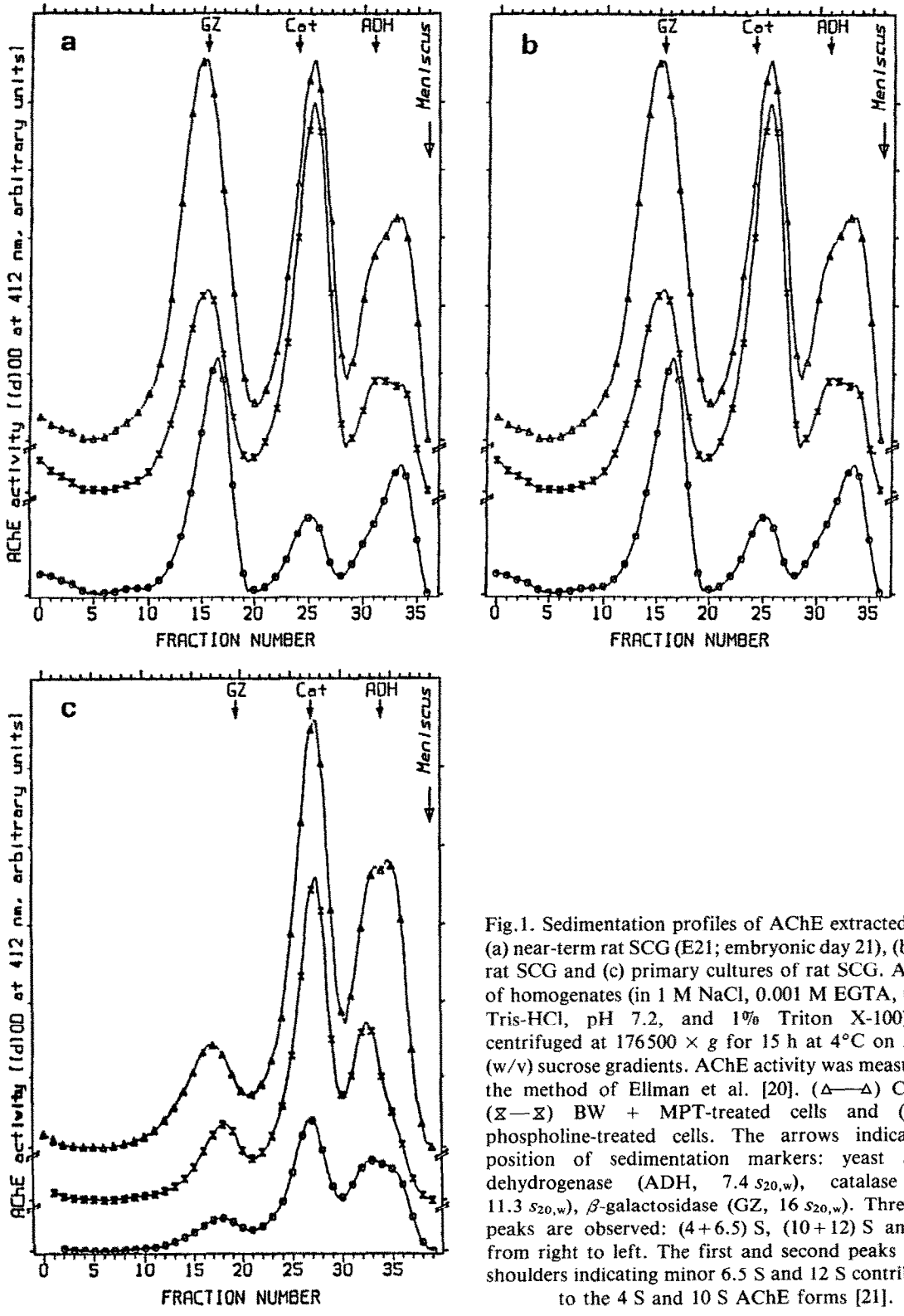

Fig.1. Sedimentation profiles of AChE extracted from: (a) near-term rat SCG (E21; embryonic day 21), (b) adult rat SCG and (c) primary cultures of rat SCG. Aliquots of homogenates (in 1 M NaCl, 0.001 M EGTA, 0.01 M Tris-HCl, pH 7.2, and 1% Triton X-100) were centrifuged at 176500 × *g* for 15 h at 4°C on 5–20% (w/v) sucrose gradients. AChE activity was measured by the method of Ellman et al. [20]. (△—△) Control, (⧖—⧖) BW + MPT-treated cells and (○—○) phospholine-treated cells. The arrows indicate the position of sedimentation markers: yeast alcohol dehydrogenase (ADH, 7.4 $s_{20,w}$), catalase (Cat, 11.3 $s_{20,w}$), β-galactosidase (GZ, 16 $s_{20,w}$). Three main peaks are observed: (4+6.5) S, (10+12) S and 16 S from right to left. The first and second peaks possess shoulders indicating minor 6.5 S and 12 S contributions to the 4 S and 10 S AChE forms [21].

Table 2

Effect of inhibitors on the activity of the different molecular forms of AChE

	AChE activity (= 100%) control			BW + MPT AChE activity (% of control)			Phospholine AChE activity (% of control)		
	(4+6.5) S	(10+12) S	16 S	(4+6.5) S	(10+12) S	16 S	(4+6.5) S	(10+12) S	16 S
E21	1.20 ± 0.04	1.69 ± 0.13	1.85 ± 0.13						
	100%	100%	100%	43.0 ± 5.4	74.3 ± 4.7	42.7 ± 4.2	70.3 ± 3.5	35.5 ± 3.5	76.1 ± 12.7
Adult	25.42 ± 2.16	29.67 ± 1.65	8.05 ± 1.14						
	100%	100%	100%	48.6 ± 3.8	67.0 ± 7.5	70.6 ± 7.8	33.7 ± 7.0	24.5 ± 5.7	39.7 ± 11.0
Culture	2.53 ± 0.26	10.49 ± 0.31	2.28 ± 0.26						
	100%	100%	100%	27.2 ± 4.8	56.9 ± 6.7	18.7 ± 2.9	116.6 ± 12.7	23.4 ± 1.3	108.3 ± 14.8

4. DISCUSSION

The aim of this work was to study the cellular localization of AChE in sympathetic neurones and characterize the extra- and intracellular pools of AChE and its main molecular forms, 4 S, 10 S and 16 S. External and internal cell compartments can be determined by the use of poorly or highly lipid-soluble, reversible or irreversible inhibitors of AChE [15]. The poorly lipid-soluble inhibitors which were chosen are phospholine and BW 284 C51. These inhibitors are not perfectly non-permeative. Consequently some quantities of these inhibitors will enter into the cell leading to approximate measurements of the extra- and intracellular pools. In the near-term SCG in vivo we found equal amounts of AChE inside and outside the cell, with 4 S and 16 S AChE essentially located inside the cell (70–75%). In contrast, 10 S AChE is prevalent outside the cell. The importance of the internal pool of4 S and 16 S AChE suggests that not only 4 S but also 16 S AChE are synthesized and preassembled intracellularly, a proposition in agreement with recent results on muscle cells [3,12,16]. In the adult SCG, AChE is slightly more extracellular than intracellular (60%). The 4 S form is equally distributed outside and inside the cell and 10 S and 16 S AChE are mostly in the external compartment (70%). In comparison with the embryonic situation there is much more 16 S AChE outside the cells in adult ganglia. Accordingly, in adult rat anterior gracilis muscle, 85% of 16 S AChE is in an extracellular environment [16,17]. In SCG, a major external location of 16 S AChE is observed only at the adult stage and may thus occur as a consequence of further postnatal maturation of the sympathetic neurones. The results obtained in vitro with primary cultures of SCG are rather different from those obtained with SCG in vivo. We found that the AChE activity is equally distributed inside and outside the cultured cells. Neuronal 10 S AChE is more important in the external compartment, as in the near-term rat SCG. But 4 S and 16 S AChE are mainly inside the cell. In our culture conditions, SCG neurones are capable of dual cholinergic-noradrenergic neurotransmitter production [18,19], and may be in a rather undifferentiated state. When adrenergic differentiation and maturation occur as it does in vivo, an increasing proportion of the cellular 4 S and 16 S AChE becomes externalized. It would be interesting to know whether the process of exter-

nalization of AChE is directly related to noradrenergic differentiation.

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